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Escherichia coli Contamination of Vegetables Grown in Soils Fertilized with Noncomposted Bovine Manure: Garden-Scale Studies

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In this study we tested the validity of the National Organic Program (NOP) requirement for a ≥120-day interval between application of noncomposted manure and harvesting of vegetables grown in manure-fertilized soil. Noncomposted bovine manure was applied to 9.3-m^2 plots at three Wisconsin sites (loamy sand, silt loam, and silty clay loam) prior to spring and summer planting of carrots, radishes, and lettuce. Soil and washed (30 s under running tap water) vegetables were analyzed for indigenous *Escherichia coli*. Within 90 days, the level of *E. coli* in manure-fertilized soil generally decreased by about 3 log CFU/g from initial levels of 4.2 to 4.4 log CFU/g. Low levels of *E. coli* generally persisted in manure-fertilized soil for more than 100 days and were detected in enriched soil from all three sites 132 to 168 days after manure application. For carrots and lettuce, at least one enrichment-negative sample was obtained ≤100 days after manure application for 63 and 88% of the treatments, respectively. The current ≥120-day limit provided an even greater likelihood of not detecting *E. coli* on carrots (≥1 enrichment-negative result for 100% of the treatments). The rapid maturation of radishes prevented conclusive evaluation of a 100- or 120-day application-to-harvest interval. The absolute absence of *E. coli* from vegetables harvested from manure-fertilized Wisconsin soils may not be ensured solely by adherence to the NOP ≥120-day limit. Unless pathogens are far better at colonizing vegetables than indigenous *E. coli* strains are, it appears that the risk of contamination for vegetables grown in Wisconsin soils would be elevated only slightly by reducing the NOP requirement to ≥100 days.

Recent scrutiny of the role of agricultural practices in contamination of fresh vegetables with pathogenic microbes (47, 54) has led to concern about the safety of using animal manures as fertilizer in vegetable production. In the North Central region of the United States, an estimated 10.4 million dairy and feedlot cattle produce between 23.6 and 35.5 kg of manure (feces and urine) per 454 kg (live weight) per day (16). Bovine manure is a good source of macro- and micronutrients, so using it as fertilizer is an important disposal method (35), particularly for organic farmers. However, bovine manure is a well-known source of food-borne pathogenic bacteria (31, 33, 41, 64, 67), and using it without prior treatment to destroy pathogens increases the likelihood of contaminating vegetables grown in manure-fertilized soils. Composting is an accepted manure pathogen reduction treatment (58), and compost-generated heat is believed to eliminate pathogenic bacteria (27, 34). However, the heat-induced death of bacteria in composted materials is a complex phenomenon (15, 28). Tailing of pathogen inactivation curves has been reported (29), as have apparent regrowth or recontamination and growth of bacteria in cooled compost (7, 38). Composting of bovine manure is not yet widely practiced in Wisconsin (1). An alternative to composting may be to passively age or store and digest the manure before application so that pathogen populations either decrease or disappear (31, 32, 37). A variety of environmental and operational variables, including storage temperature (26) and the diet of the cattle (37), could affect the extent of pathogen decrease, and complete elimination of pathogens is not assured by this technique (20). Further death of manure-borne pathogens occurs once the manure is incorporated into the soil. Weather conditions, desiccation, soil type, predatory protozoan populations, and the degree of manure incorporation are all likely to have various effects on pathogen survival in manure-fertilized soil (5, 6, 8, 9, 18, 19, 21, 36, 40, 42, 51–53, 59, 66). Because of the wide range of variables associated with manure composition, preapplication storage, application, and incorporation, the United States Department of Agriculture National Organic Program (NOP) specifies a minimum manure application-to-harvest time interval necessary to provide adequate assurance of safety. To prevent manure-borne pathogen contamination of vegetables, the NOP regulations require at least 120 days between application of noncomposted manure and the harvesting of organic crops that have edible portions exposed to soil particles (58). Previous studies in which controlled-environment chambers and soil beds were used suggested that an application-to-harvest interval of less than 120 days might be safe in certain Wisconsin situations (39). These studies, along with the studies of other workers, validated the use of indigenous *Escherichia coli* as a surrogate for pathogenic *E. coli* O157:H7 (40) and *Salmonella* spp. (39). Subsequent field studies (unpublished data) suggested that indigenous *E. coli* may die faster in manure-fertilized soil under field conditions than in soil beds and resulted in the hypothesis that a 90-day application-to-harvest interval would adequately minimize the risk of contamination of leaf and root crops. The present field study was done to test this hypothesis

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TABLE 1. Physical and chemical characteristics of soils

Characteristic	Soil type (location)		
	Loamy sand (Hancock)	Silt loam (Lancaster)	Silty clay loam (West Madison)
% Sand	87	12	19
% Silt	6	75	65
% Clay	7	13	16
pH	6.8	6.0	6.3
% Organic matter	1.0	2.7	3.8
% Total N	0.09	0.14	0.18
Available P (ppm)	135	24	30
Available K (ppm)	118	118	132

in three different Wisconsin soil types by using garden-scale, low-chemical-input agricultural practices.

MATERIALS AND METHODS

Field sites: soil characteristics, preparation, and layout. The field study was conducted at University of Wisconsin-Madison agricultural research stations at Hancock, Lancaster, and West Madison, Wis., during the summer of 2003. Prior to the study, each field was cropped in alfalfa. A wide-spectrum herbicide (RoundUp Ultra; Monsanto, St. Louis, Mo.) was applied to clear the field in the fall of 2002. The soil characteristics of each field site were determined by analyses at the University of Wisconsin-Madison Soil and Plant Analysis Laboratory and are shown in Table 1. The soil classifications were loamy sand (Hancock), silt loam (Lancaster), and silty clay loam (West Madison). Each field site consisted of a 4 × 11 grid of treatment plots that were 3.05 by 3.05 m (a total of 44 plots) with a 4.6-m alley on each side of each plot. In the spring of 2003, white clover was planted in the alleys to control weed growth and to minimize lateral soil transfer during rainstorms. Each treatment was randomly assigned to two plots at each site. The variables considered in treatments were the date of manure application; the time of planting; whether manure was immediately tilled into the soil, tilled into the soil immediately prior to planting, or not tilled into the soil; and whether the manure was amended with chopped oat straw. Although manure is typically tilled into the soil within a few days after application (and in some cases prompt tilling is required by environmental regulations), manure is sometimes left on top of the soil for lengthy periods before tilling, due to weather conditions or more pressing work demands. Amendment with straw is not a common intentional practice, but various amounts of straw may be present in bovine manure if straw is used as bedding. The purpose of adding straw in the present study was to possibly stimulate predatory protozoan activity. Descriptions of key treatments for each site are shown in Table 2.

Bovine manure application. Fresh manure (≤3 days old) was collected at the University of Wisconsin-Madison Dairy Cattle Instruction and Research Center from a herd of lactating Holstein cows fed a standard corn- and soy-based diet. In order to maximize the levels of indigenous *E. coli*, care was taken to obtain manure with low levels of bedding material. Manure was shoveled into 19.4-liter (5-gallon) buckets (approximately 16 kg per bucket), and the buckets were

TABLE 2. Survival of indigenous *E. coli* in three soils fertilized with noncomposted bovine manure

Soil (location)	Treatment no.	Manure application date (day/mo)[a]	Date of tilling (day/mo)[a]	Inoculum level[b]	No. of days for:		
					First plot enrichment[c]	Second plot enrichment done	First enrichment negative[d]
Loamy sand (Hancock)	Control 1	None[e]	None	4.8	198	209	198
	Control 2	None[e]	None	4.9	198	198	198
	1	4/21	4/21	4.1	71	71	100
	3	4/23	5/13	4.3	54	54	126
	5	5/13	5/13	4.7	49	59	118
	7	5/13	None	2.6	34	78	>132[f]
	9	6/11	6/11	4.9	NT[g]	NT	>103[f]
	11	6/11	7/9	5.9	NT	NT	>103[f]
Silt loam (Lancaster)	Control 1	None[e]	None	4.3	182	182	182
	Control 2	None[e]	None	4.3	182	182	182
	1	4/14	4/14	2.9	84	84	128
	3	4/18	5/21	4.2	80	95	110
	5	5/19	5/28	4.3	79	79	>133[f]
	7	5/8	None	4.3	60	104	90
	9	6/4	6/4	4.9	63	77	>117[f]
	11	6/4	None	4.9	33	48	>117[f]
Silty clay loam (West Madison)	Control 1	None[h]	None	<1.2[i]	28	28	42
	Control 2	None[h]	None	<1.3[i]	28	28	42
	1	4/15	4/15	4.0	83	98	>167[f]
	3	4/16	5/21	4.3	41	41	41
	5	5/13	5/13	4.3	70	83	>113[f]
	7	5/8	None	4.4	45	45	>133[f]
	9	6/2	6/2	4.3	35	79	>93[f]
	11	6/2	6/13	4.8	50	79	>119[f]

[a] All dates are in 2003.
[b] Mean log CFU per gram of manure-fertilized soil for two plots.
[c] Number of days after manure application when enrichment was done for at least one plot (the preceding sample from the plot yielded no presumptive *E. coli* colonies as determined by direct plating [detection limits, approximately 0.9 to 1.4, 1.1 to 1.9, and 1.0 to 1.6 log CFU/g for loamy sand, silt loam, and silty clay loam, respectively]).
[d] Number of days after manure application when a plot for a treatment analyzed by enrichment first yielded negative results.
[e] Manure was applied during the preceding fall (2002).
[f] No enrichment-negative results were obtained; no sampling was done after the number of days indicated.
[g] NT, no direct plating done. The enrichment procedure was performed for the first samples after manure application and thereafter.
[h] No manure was applied during the preceding fall or in the spring of 2003. The times for first enrichment, second enrichment, and first enrichment negative are the numbers of days since the initial sampling.
[i] No presumptive *E. coli* was detected by direct plating for initial samples from both plots.

TABLE 3. Indigenous *E. coli* on carrots, lettuce, and radishes grown in three soils fertilized with noncomposted bovine manure

Soil (location)	Treatment no.	Manure application date (day/mo)[a]	Carrots		Lettuce		Radishes	
			Planting date (day/mo)[a]	First enrichment negative (days)[b]	Planting date (day/mo)[a]	First enrichment negative (days)[b]	Planting date (day/mo)[a]	First enrichment negative (days)[b]
Loamy sand (Hancock)	Control 2	None[c]	5/8	244	5/12	244	5/12	244
	1	4/21	5/12	91	5/12	63	5/12	>82[d]
	3	4/23	5/16	107	5/28	>80[d]	5/16	>77[d]
	5	5/13	5/30	69	5/30	60	5/30	61
	7	5/13	5/29	69	5/29	>78[d]	5/29	>61[d]
	9	6/11	7/9	103	7/9	77	7/9	>77
	11	6/11	7/9	89	7/9	77	7/9	>77[d]
Silt loam (Lancaster)	Control 2	None[c]	5/21	288	5/21	265	5/21	240
	1	4/14	5/21	120	5/21	97	5/21	72
	3	4/18	5/21	116	5/21	93	5/21	68
	5	5/19	5/28	85	5/28	62	5/28	>64[d]
	7	5/8	5/28	96	5/28	84	5/28	>75
	9	6/4	6/17	NC[e]	6/17	NC	6/17	NC
	11	6/4	6/17	NC	6/17	NC	6/17	NC
Silty clay loam (West Madison)	Control 2	None[f]	5/27	119	5/27	113	5/27	98
	1	4/15	5/22	104	5/22	98	5/22	69
	3	4/16	5/22	103	5/22	97	5/22	68
	5	5/13	6/5	76	5/27	70	5/27	>55[d]
	7	5/8	6/5	81	5/27	75	5/27	46
	9	6/2	6/17	94	6/17	56	6/17	56
	11	6/2	6/17	94	6/17	56	6/17	56

[a] All dates are in 2003.
[b] Number of days after manure application when a plot for a treatment first yielded a washed vegetable sample that was negative following enrichment.
[c] Manure was applied during the preceding fall (2002).
[d] No enrichment-negative results were obtained; no sampling was done after the number of days indicated.
[e] NC, no crop harvested, because of drought.
[f] No manure was applied during the preceding fall or in the spring of 2003. The times for first enrichment negative are the numbers of days since the initial sampling.

transported within 1.5 h to the field sites. The manure was applied at a rate of five buckets (80 kg) per treatment plot to simulate a typical manure application rate in Wisconsin, 67.2 metric tons (wet weight) per ha. To apply the manure, the buckets were emptied onto the treatment plot soil, and the manure was distributed evenly over the soil surface with a shovel. Some treatments included application of 0.5 bale (ca. 7 kg) of chopped oat straw to the treatment plot before manure addition. For these treatments, the straw was manually distributed over the soil surface, and this was followed by manure application and spreading. The plots treated with manure or with manure and straw were tilled with a shovel either immediately after application, immediately before planting, or not at all. Tilling with a shovel incorporated the manure to a depth of about 15 cm.

Vegetable production. Each treatment plot (other than controls) was manually planted with two side-by-side rows each of carrot (cultivar Short 'n' Sweet; W. Atlee Burpee & Co., Warminster, Pa.), radish (cultivar Cherry Belle; Burpee), and lettuce (cultivar Simpson Elite; Veseys Seeds, Ltd., Charlottetown, Prince Edward Island, Canada). There were two rows of each vegetable for a total of six rows; each row was about 2.7 m long and 46 cm from each neighboring row. At each site the rows were oriented north-south. The rates of planting were 2.0, 4.0, and 1.2 g of seeds per row for carrot, radish, and lettuce, respectively. Planting was done in May and June (Table 3). The vegetable plots on loamy sand were irrigated twice weekly from June through mid-September at a rate of 1.27 cm per irrigation. The total additional water was 40 cm, and the rainfall data and the daily high and low temperatures are shown in Tables 4 and 5, respectively. No irrigation was available for the silt loam, and vegetable growth ceased during a prolonged drought beginning in July and continuing for the remainder of the summer (Table 4). The silty clay loam was irrigated as needed, and the irrigation data and weather data are shown in Tables 4 and 5.

Soil sampling and analysis. Soil samples from each treatment plot were analyzed at biweekly intervals, beginning at the time of manure application. A composite of three soil core samples was collected from each treatment plot by using one sterile aluminum soil corer (Forestry Suppliers, Jackson, Miss.), one sterile tongue depressor to push the soil out of the corer, and one sterile stomacher filter bag (Nasco, Ft. Atkinson, Wis.). To determine the sampling location within each plot, the plot was divided into quadrants, a quadrant was randomly selected, and three samples were taken from randomly chosen locations within the quadrant. Soil samples were placed in shaded insulated coolers, transported to the laboratory, and refrigerated (5°C) until analysis. Each soil sample was weighed, and then 198 ml of Butterfield's phosphate diluent (BPD) (Nelson Jameson, Marshfield, Wis.) was added. The diluted sample was then manually shaken for 30 s, allowed to sit for 30 s, and then manually shaken for another 30 s. Decimal serial dilutions in BPD were prepared, and 1.0 ml of a dilution was plated on a 3M Petrifilm *E. coli*-coliform count plate (3M Microbiology Products, St. Paul, Minn.). The initial sample dilution (in the stomacher bag) was not plated because the soil color obscured colonies on the plate. The plates were incubated at 35°C for 48 h, and then presumptive *E. coli* colonies (blue with associated gas) were counted. By using the soil sample weight, dilution factor, and number of presumptive colonies, the log number of CFU per gram of soil was calculated for each treatment plot, and then the mean log number of CFU per gram for each treatment was calculated from values for the log number of CFU per gram for the duplicate treatment plots. When no colonies were detected for the least diluted sample plated, a log number of CFU per gram was calculated from an arbitrarily assigned value (0.5 colony).

When a soil sample yielded no presumptive *E. coli* colonies, an enrichment procedure was used for that treatment plot at the next sampling time. In this procedure, the initial dilution and mixing were performed by using nutrient broth (Difco, Becton Dickinson, Sparks, Md.) instead of BPD. For nonselective enrichment, the initial dilution was incubated for 24 h at 35°C. Then the initial dilution was gently mixed, and 1.0 ml was transferred to 9 ml of lauryl tryptose broth (Difco), which was vortex mixed and incubated at 45.5°C for 24 h as a selective enrichment step. Next, the contents of the selective enrichment tube were vortexed, and one loopful was streaked onto Levine eosin methylene blue agar (LEMB) (Difco). Each LEMB plate was incubated for 24 h at 35°C and observed for colonies with dark centers, with or without an associated metallic sheen. A positive result was recorded if one or more such colonies were present. For confirmation, presumptive colonies from Petrifilm *E. coli*-coliform count and LEMB plates were streaked onto brain heart infusion agar (Difco) and incubated 24 h at 35°C, and then an isolated colony was tested to determine its cell morphology, Gram reaction, oxidase reaction, and biochemical characteristics

TABLE 4. Weekly precipitation and irrigation amounts at the Hancock (loamy sand), Lancaster (silt loam), and West Madison (silty clay loam) sites during the study

Study week	Date	Hancock		Lancaster		West Madison	
		Precipitation (mm)	Irrigation (mm)	Precipitation (mm)	Irrigation (mm)	Precipitation (mm)	Irrigation (mm)
1	1–7 April	1	0	3	0	1	0
2		0	0	1	0	0	0
3		4	0	4	0	3	0
4		0	0	0	0	0	0
5		5	0	22	0	14	0
6		10	0	15	0	9	0
7		2	0	9	0	11	0
8		0	0	8	0	1	0
9		2	0	4	0	4	0
10	3–9 June	8	25	7	0	6	0
11		1	25	1	0	1	0
12		1	25	0	0	10	19
13		5	25	13	0	8	0
14		3	25	9	0	11	0
15		4	25	8	0	8	0
16		0	25	1	0	10	0
17		0	25	1	0	0	0
18		2	25	1	0	1	19
19		1	25	1	0	0	0
20	12–18 August	0	25	0	0	1	19
21		1	25	3	0	2	19
22		0	25	7	0	7	19
23		0	25	0	0	0	0
24		8	25	19	0	21	0
25		1	25	6	0	2	0
26		1	0	3	0	0	0
27		1	0	0	0	1	0
28		5	0	8	0	8	0
29		0	0	16	0	7	0
30	21–27 October	1	0	11	0	12	0
Total		67	400[a]	181	0[a]	159	95[a]

[a] The total amounts (precipitation plus irrigation) for the Hancock, Lancaster, and West Madison soils were 467, 181, and 254 mm, respectively.

(API 20E kit; BioMerieux, Hazelwood, Mo.). For the first 3 months of the study, one colony from a given treatment at a given sampling time (e.g., one colony each from treatments 1, 2, 3, and 4 at the 11 April sampling) was tested to confirm the identity. After this, one colony was tested for each combination of manure application date and tilling (e.g., treatments 1 and 3 at an August sampling date). Colonies from treatments that received manure and straw were not tested after the first 3 months. Over the course of the study, 85% (271 of 319) of presumptive *E. coli* isolates were conclusively confirmed to be *E. coli*, while 8.5% were identified as doubtful *E. coli*; the remaining isolates were identified as various coliforms (*Klebsiella*, *Citrobacter*, *Enterobacter*), *Kluyvera*, *Pasteurella*, and *Serratia*. Because presumptive counts would always be greater than or equal to confirmed counts (thus overestimating *E. coli* survival), additional colonies were not tested when a colony was not confirmed to be *E. coli*.

Vegetable sampling and analysis. Vegetable samples were obtained at thinning and harvest. Vegetables were randomly selected from the plants thinned or harvested, and the edible portions (roots for radish and carrot and leaves for lettuce) were separated by using scissors or shears previously sprayed with 70% ethanol, placed in clean plastic sealable bags, and transported to the laboratory in insulated coolers. In the laboratory, a 25-g random subsample for a given treatment plot was weighed on cheesecloth. The cheesecloth and vegetables were placed into a colander that had been previously sprayed with 70% ethanol, and the vegetables were washed with cool running tap water (3.3 liters per min) for 30 s. After draining for 1 min, the vegetables were aseptically transferred to a sterile filter whirlpack bag for stomaching. Vegetables that were too large for stomaching were first cut into smaller pieces with a knife that had previously been sprayed with 70% ethanol. To the vegetables, 99 ml of nutrient broth was added, and the mixture was stomached on medium speed for 30 s. The samples were then analyzed by direct plating and enrichment by using the methods described above. The confirmation rate for presumptive *E. coli* isolates was 64% (145 of 225 isolates were confirmed to be *E. coli*); 20% of the isolates were identified as doubtful *E. coli*, 5% each were identified as *Enterobacter* and *Klebsiella*, and the remaining isolates were identified as *Kluyvera*, *Citrobacter*, *Serratia*, and *Kleviscia*.

Presentation of data and statistical analysis. All data were organized by treatment and are expressed below in terms of the number of days following manure application. Treatments were empirically compared in terms of first plot enrichment (the number of days after manure application when enrichment was first done for at least one plot [i.e., the preceding sample from that plot yielded no presumptive *E. coli* colonies as determined by direct plating]), second plot enrichment, and first negative enrichment (the number of days after manure application when a plot for a given treatment first yielded a negative result following enrichment). Analysis of variance (Minitab, release 12; Minitab, Inc., State College, Pa.) with a 5% significance level was used to compare soils in terms of mean first plot enrichment time, second plot enrichment time, and time until the first enrichment-negative result for soil and each vegetable. Similar analyses were done to compare treatments in which manure was tilled into the soil immediately to treatments with delayed tilling and treatments with no tilling. When no enrichment-negative result was obtained, 14 days was added to the time for the final enrichment-positive result, and this value was used in the statistical analyses.

RESULTS

In general, the levels of indigenous *E. coli* decreased rapidly in the manure-fertilized soils (Table 2). Statistical comparisons of the intervals between the time of manure application and the time at which one or both plots for each treatment were analyzed by enrichment showed that the mean time intervals did not differ significantly between soils ($P > 0.05$). For three

TABLE 5. Average weekly high and low temperatures at the Hancock (loamy sand), Lancaster (silt loam), and West Madison (silty clay loam) sites during the study

Study week	Date	Avg temp (°C)[a]					
		Hancock		Lancaster		West Madison	
		High	Low	High	Low	High	Low
1	1–7 April	3	−5	11	−2	5	−3
2		17	−1	13	−1	16	0
3		12	−1	17	6	15	5
4		18	3	18	4	18	4
5		14	5	16	6	14	6
6		18	7	17	7	18	9
7		19	9	19	9	19	9
8		19	5	19	7	19	7
9		22	9	23	10	21	9
10	3–9 June	21	11	20	10	21	12
11		24	12	25	14	24	14
12		27	12	26	14	26	13
13		25	14	26	15	26	16
14		29	17	29	19	30	18
15		23	14	24	15	23	15
16		26	13	27	15	26	16
17		27	15	26	16	26	17
18		27	15	28	16	26	16
19		27	14	27	15	26	16
20	12–18 August	30	17	30	17	29	18
21		31	18	31	18	31	19
22		27	11	29	14	26	14
23		28	13	27	12	27	15
24		25	14	26	15	24	15
25		24	10	23	14	24	11
26		14	5	17	4	16	6
27		13	1	13	0	13	1
28		23	9	23	9	23	10
29		17	3	16	3	17	5
30	21–27 October	10	−1	14	2	11	2

[a] Averages were calculated from the daily high and low temperatures for each 7-day period.

of four treatments on loamy sand, the first plot was analyzed by enrichment after <63 days. For these four treatments, the second plot was analyzed by enrichment in <63 days for two treatments and after 71 and 78 days for the remaining two treatments. For three of six treatments on the silt loam, the first plot was analyzed by enrichment after ≤63 days, and for the remaining three treatments the first plot was analyzed by enrichment after 79 to 84 days. However, for one of the six treatments the second plot was analyzed by enrichment after <63 days, and for two treatments the second plot was not analyzed by enrichment until ≥95 days. Intermediate results were obtained for the silty clay loam. The treatment-to-treatment differences between soils in terms of indigenous *E. coli* survival may have resulted from the more rapid drainage and drying in the loamy sand or from differences in the frequency and amount of rainfall and irrigation (Table 4). Interestingly, the silty clay loam empirically differed from the other two soils in the length of time between manure application and the first enrichment-negative result (Table 2) despite the lack of a statistically significant difference between soils. For silty clay loam, no enrichment-negative results were obtained for five of the six treatments. In contrast, enrichment-negative results for the silt loam were obtained in ≤90 days for one treatment, in 110 to 128 days for two treatments, and not at all for three treatments. For loamy sand, the first enrichment-negative results were obtained for one treatment in 100 days, the first enrichment-negative results were obtained for two treatments in 118 to 126 days, and no enrichment-negative results were obtained for three treatments. Low levels of *E. coli* generally persisted in manure-fertilized soil for well over 100 days and were detected in enriched soil from all three sites 132 to 168 days after manure application.

There was considerable variability with no statistically significant differences between soils or between application-to-tilling intervals in terms of first enrichment, second enrichment, and first enrichment-negative times. The addition of chopped oat straw to the manure applied had no apparent effect on indigenous *E. coli* survival for any soil type. Because this practice resulted in more difficult tilling and increased weed growth, it cannot be recommended. For the sake of brevity, results from added-straw treatments are not shown here.

Low-level contamination (direct plating negative, enrichment positive) with indigenous *E. coli* occurred sporadically for washed carrots, lettuce, and radishes regardless of whether manure had been applied. Nevertheless, enrichment-negative results were common for carrots and lettuce harvested <120 days after manure application. For washed carrots, six of six, four of four, and six of six treatments yielded enrichment-negative results for samples from loamy sand, silt loam, and silty clay loam, respectively, within 120 days after manure application (Table 3). For four of six, two of four, and four of six treatments, respectively, on the three soils, enrichment-negative results were obtained for washed carrots harvested ≤100 days after manure application. Occasional enrichment-positive results were obtained for washed carrots harvested 128 and 133 days after manure application for samples from loamy sand and silty clay loam soils, respectively.

Contamination of lettuce was less likely to occur than contamination of carrots. For most treatments for the three soils, enrichment-negative results were obtained for lettuce harvested within 100 days after manure application; the exceptions were two loamy sand treatments in which the lettuce bolted soon after enrichment-positive results at 78 to 80 days after manure application (Table 3). Most lettuce bolted before the 120-day application-to-harvest limit could be tested, but sporadic enrichment-positive results were obtained for lettuce harvested from the silty clay loam 120 to 121 days after manure application.

The results for radishes were more ambiguous because of rapid radish maturation. On loamy sand, only one of six treatments resulted in enrichment-negative results for washed radishes harvested ≤77 days after manure application (Table 3). The five other treatments did not yield enrichment-negative results by the time of the final harvest (61 to 82 days after manure application). Similar results were obtained for radishes grown in silt loam soil. For radishes grown on silty clay loam, five of six treatments resulted in enrichment-negative results within ≤69 days after manure application. Thus, an application-to-harvest interval of ≥100 or 120 days for radishes could not be effectively evaluated. There was no statistically significant difference between soils or between manure application-to-tilling intervals in terms of time before enrichment-negative results were obtained for any type of vegetable.

For all of the field sites combined, indigenous *E. coli* was

detected by direct plating of washed vegetables for only 5 of 38, 1 of 48, and 6 of 24 samples throughout the study for carrots, lettuce, and radishes, respectively. For the 12 direct plating-positive results, the mean level of indigenous *E. coli* detected was less than 1.0 log CFU/g for all but one treatment for carrots (loamy sand treatment with June manure application and harvest 89 days later) and two treatments for radishes (loamy sand with May manure application and harvest 41 days later and loamy sand with June manure application and harvest 58 days later). The mean levels of indigenous *E. coli* in these cases were 1.1 to 1.2 log CFU/g.

DISCUSSION

The rate of decrease in the *E. coli* level observed in manure-fertilized soils in this study was within the range of bacterial death rates in soil or manure-fertilized soils reported elsewhere for microcosm and field studies. Guo et al. (23) observed a 1- to 1.5-log decrease in the levels of salmonellae in inoculated topsoil during 45 days of 20°C storage in greenhouse trays. The levels of a nonpathogenic *E. coli* strain decreased by 6 log CFU/g in 60 days at 4 and 20°C and in 12 days at 37°C in nonsterile sieved soil in flasks (4). Recorbet et al. (42) reported that *E. coli* death in inoculated soil microcosms stored at 28°C was dose dependent, with 3- to 4-log CFU/g decreases occurring within 5 days. In a field study involving application of contaminated pig slurry (feces plus urine) to soil, Baloda et al. (2) detected *Salmonella enterica* serovar Typhimurium DT12 in treated soil 14 days after manure application but not 21 days after application. Stoddard et al. (51) reported a 6.9-day half-life for fecal coliforms in bovine manure-fertilized soil, although the rate of decline decreased with time. Van Donsel et al. (59) reported that fecal coliform levels in manure-fertilized soils decreased by 3 log CFU/g in 2 to 4 weeks, depending on the ambient temperature and the exposure to direct sunlight. Similar trends were reported by Tannock and Smith (53) for salmonellae.

For all three manure-fertilized soils in this study, persistence of indigenous *E. coli* past the end of the study was common. Persistence of fecal bacteria in soil has been reported elsewhere. For example, Jones (30) described *E. coli* that survived for at least 60 days in soil at 25°C and for at least 100 days at 4°C. Bolton et al. (5) detected *E. coli* O157:H7 in soil 99 days after a fecal suspension containing this organism was applied to grassland. It is possible that fecal bacteria may enter a viable but nonculturable state in soil after an extended time (56), although it is uncertain whether pathogenic bacteria in this state would be infective. The persistence of *E. coli* in the present study was likely either the result of bird and mammal defecation or movement within the plots (tracks and droppings were observed at all three locations, with greater prevalence on the silt loam and silty clay loam soils) or the result of protection of bacteria within soil particles. In control plots to which manure had been applied the preceding fall (2002; loamy sand and silt loam), sporadic enrichment-positive results were obtained throughout the 2003 growing season. When no manure was applied in either the fall of 2002 or the spring of 2003 (silty clay loam), enrichment-negative results were obtained early in the spring of 2003 and throughout the 2003 growing season. However, these enrichment-negative results were interspersed with enrichment-positive results, suggesting that bird and/or mammal recontamination was the cause of the apparent persistence of indigenous *E. coli* in manure-fertilized soils. Together, the results of the manure-fertilized soil analyses suggested that application of noncomposted bovine manure far enough in advance of vegetable harvest would present little additional risk beyond that associated with nonfertilized soil. Furthermore, our observations and data suggested that bird and mammal activity may result in contamination of vegetable production soils regardless of when or if manure is used as fertilizer.

In our study, sporadic low-level *E. coli* contamination of vegetables occurred even when the NOP $\geq$120-day limit was followed. In light of previously published results for colonization of vegetables by pathogenic bacteria, these results are not surprising. In a study of vegetables grown in inoculated soil under greenhouse conditions, Van Renterghem et al. (60) found that *Listeria monocytogenes*, previously added to soil at a level of 5 log CFU/g, was detected by enrichment on three of six radish samples 3 months after inoculation. In contrast, none of six carrot samples tested positive and only one of six samples of radish soils tested positive. Although the numbers of samples were low, these results suggest that the contamination rates for different vegetables may vary under identical growing conditions and that vegetables may become contaminated with soilborne pathogens early in growth. Several studies have suggested that colonization of plants is most likely when the plant is a seedling, and the emerging root is a key area of bacterial attachment (11, 14). Subsequent internalization of soilborne pathogenic bacteria is also greatest when the plants are seedlings, and internalization is less likely as the plants mature (11). The ability to colonize vegetable roots and shoots varies for different strains of a bacterial species (3, 62) and for different bacterial species (3, 10, 14). Bacterial motility (11) and the ability to use seed exudates as carbon sources (45) are related to the extent of colonization. Internalization of bacteria in seedlings is more likely in hydroponic systems than in soil (24, 63), but internalization and transport throughout the plant can still occur in the latter growth medium (49). Collectively, the literature on bacterial colonization of plants and internalization suggests that the critical time for preventing vegetable contamination with manure fertilizer may be at the time of planting. A better alternative to an application-to-harvest interval may be an application-to-planting interval.

Enrichment-negative results were obtained more often for vegetables than for soil samples. The possible causes of this trend included recontamination of soil, but not vegetables, by birds and other animals, competitive exclusion of *E. coli* by other microbes on the vegetable surface, and release of antimicrobial plant-derived compounds when samples were stomached prior to enrichment.

Examination of our results leads to two seemingly contradictory conclusions about the current NOP 120-day application-to-harvest interval. One conclusion, based on the persistence of indigenous *E. coli* in the soil and the occasional detection of indigenous *E. coli* on carrots harvested >120 days after manure application, is that even the current 120-day limit does not completely guarantee the absence of contamination. The second conclusion is that decreasing the 120-day limit to 100 days would only slightly increase the risk of contamination.

Although the prevalence and shedding of pathogenic *E. coli* in cattle may be affected to some extent by dietary changes (12, 13, 25, 46), it is apparent from studies of herds (17, 43, 55, 65) and slaughterhouses (44, 57) that carriage of *E. coli* O157:H7, other Shiga toxin-producing *E. coli*, and *Salmonella* spp. within herds is common. Furthermore, some research suggests that pathogenic *E. coli* can persist within a cattle farm for several years (48). Therefore, it is prudent to assume that any noncomposted bovine manure applied to vegetable production fields contains these pathogens. However, the sporadic pathogen carriage and shedding for most animals in a herd (22) should result in lower levels of pathogenic bacteria than of indigenous *E. coli* in bovine manure. Because indigenous *E. coli* has been shown to behave like the two main enteric bacteria that cause vegetable-associated outbreaks (39, 40), it could be argued that a large reduction in the level of indigenous *E. coli* in manure-fertilized soil that occurred within a ≥100-day application-to-harvest interval should result in a satisfactorily low likelihood of vegetable contamination by manure-borne *E. coli* O157:H7 and *Salmonella* spp. However, it is also possible that some pathogens more readily colonize vegetables and/or survive in manure-fertilized soils than indigenous *E. coli* does. In the present study, reductions in the indigenous *E. coli* levels of at least 3 log CFU/g of manure-fertilized soil were readily achieved within this application-to-harvest interval. For lettuce and carrots grown in the present study, the combination of a 100-day application-to-harvest interval and passive washing for 30 s under running tap water resulted in frequent enrichment-negative results when vegetables were tested for indigenous *E. coli*. The two-step enrichment procedure used to detect *E. coli* was very sensitive; a detection limit of approximately one cell was determined in supplemental, broth-based experiments (data not shown). However, the ability of pathogens to grow to detectable levels during this procedure was not tested. Given the sensitivity of the two-step enrichment method, the presumed greater numbers of indigenous *E. coli* than of enteric pathogenic bacteria in bovine manure, and the gentleness of the washing treatment, a 100-day application-to-harvest interval may result in a satisfactory reduction in the risk of pathogen contamination.

An appropriate manure application-to-harvest interval cannot be a stand-alone guarantee against vegetable contamination. Agricultural practices other than fertilization with manure can result in transfer of pathogenic bacteria from soil to vegetables. For example, Wachtel et al. described *E. coli* O157:H7 contamination of vegetable roots via soil after the soil was irrigated with contaminated water in laboratory (61) and field (62) studies. Irrigation with contaminated irrigation water may also lead to pathogen colonization of vegetable leaves (50). Furthermore, unclean conditions may contaminate vegetables after they are harvested (54). Therefore, use of a 100-day application-to-harvest interval would be contingent on thorough washing and strict adherence to good agricultural practices.

In conclusion, examination of our results suggests that using the current NOP manure application-to-harvest interval of ≥120 days does not guarantee the absence of manure-borne bacteria from vegetables grown in manure-fertilized Wisconsin soils. However, we concluded that decreasing the NOP manure application-to-harvest interval from ≥120 to ≥100 days for typical Wisconsin soils would only slightly increase the risk of vegetable contamination. Such a decrease would provide Wisconsin vegetable farmers with greater flexibility in scheduling manure application and would lessen the likelihood of soil compaction caused by manure application too early in the spring. Other agricultural factors, such as the application-to-planting interval, harvest hygiene, and postharvest washing treatments, are likely also critically important in preventing contamination of vegetables with pathogenic bacteria. The findings of the present study should tested in field studies with manure-borne pathogens.

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